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The Tunica Adventitia of Human Arteries and Veins As a Source of Mesenchymal Stem Cells

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We previously demonstrated that human pericytes, which encircle capillaries and microvessels, give rise in culture to genuine mesenchymal stem cells (MSCs). This raised the question as to whether all MSC are derived from pericytes. Pericytes and other cells defined on differential expression of CD34, CD31, and CD146 were sorted from the stromal vascular fraction of human white adipose tissue. Besides pericytes, CD34+ CD31- CD146- CD45- cells, which reside in the outmost layer of blood vessels, the tunica adventitia, natively expressed MSC markers and gave rise in culture to clonogenic multipotent progenitors identical to standard bone marrow-derived MSC. Despite common MSC features and developmental properties, adventitial cells and pericytes retain distinct phenotypes and genotypes through culture. However, in the presence of growth factors involved in vascular remodeling, adventitial cells acquire a pericytes-like phenotype. In conclusion, we demonstrate the co-existence of 2 separate perivascular MSC progenitors: pericytes in capillaries and microvessels and adventitial cells around larger vessels.

Introduction

The in vitro generated mesenchymal stem cell (MSC) experimentally differentiates into mesodermal lineage cells and can modulate some immune responses [1,2]. Initially extracted from the bone marrow, MSC can be derived from most if not all organs, which may reflect the existence of a systemic reservoir of multipotent progenitor cells [3–5]. However, the in vivo counterpart of the artificial MSC is still not fully characterized [6–8]. An affiliation between MSC and vascular cells has been suggested by the isolation of MSC from artery or vein walls and, less directly, by the correlation between MSC progenitor frequency and vessel density in equine adipose tissue [9]. In the bone marrow, CD146+ reticular cells lining the endothelium in sinusoid walls can self-renew, differentiate into bone, and recapitulate the hematopoietic microenvironment in vivo [10]. Similarities between MSC and perivascular cells have been also described in the dental pulp, endometrium, and several other tissues [11–14]. We recently demonstrated a perivascular origin of human MSC, thus showing that pericytes purified from multiple organs natively display phenotypic and developmental features of MSC [15]. Moreover, cultured pericytes resemble MSC by morphology and growth properties, retain the expression of MSC surface markers, and can differentiate into bone, cartilage, fat, and muscle cells, similar to MSC [15]. Although MSC can clearly derive from cultured pericytes, there is no argument to exclude that other cell types, including other vascular cells, are also at the origin of MSC [16]. In the current study, we investigated whether other cells share with pericytes the ability to originate MSC. To address this question, we purified different subsets of cells distinct from pericytes from the stromal vascular fraction of human white adipose tissue (hWAT) and tested their ability to yield MSC in culture. We here report the identification of a novel perivascular MSC progenitor, typified as CD34+CD31-CD146-CD45- and located in the tunica adventitia of arteries and veins in multiple human tissues, hence distinct from pericytes. Although the presence of multipotent progenitors in the tunica adventitia has been suggested by several studies [17–29], ignorance of their antigenic phenotype has precluded the isolation and characterization of these progenitors from human organs.

Materials and Methods

Human tissues

Human adipose tissue surgical specimens ($n=18$) and lipoaspirates ($n=15$) were obtained from female patients undergoing abdominoplasty and liposuction, respectively.

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Specimens were obtained as anonymous and unidentified samples; no IRB approval was, therefore, required. Human fetal tissues were obtained after spontaneous, voluntary, or therapeutic pregnancy interruptions performed at Magee-Womens Hospital (University of Pittsburgh), in compliance with Institutional Review Board protocol number 0506176.

Cell sorting

The stromal vascular fraction of hWAT was analyzed by flow cytometry, and different populations of cells were sorted. Surgical hWAT was minced, whereas lipoaspirate was diluted with an equal volume of PBS before digestion with Dulbecco's modified Eagle's medium (DMEM) containing 3.5% bovine serum albumin (Sigma) and collagenase II (1 mg/mL; Sigma) for 70 min under agitation at 37°C. Adipocytes were separated by centrifugation and removed. Pellets were resuspended in erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA) and incubated for 10 min at room temperature. After centrifugation, pellets were resuspended in PBS and passed through a 70-μm cell filter (BD Biosciences). Fetal tissues were processed as previously described [15]. Cells were incubated with uncoupled anti-CD31 (DAKO; 1:50), then with biotinylated goat anti-mouse Ig (DAKO; 1:500) followed by incubation with streptavidin-PE-Texas Red (BD Biosciences; 1:500). Cells were eventually incubated with a mixture of the following directly conjugated antibodies: anti-CD34-PE (DAKO; 1:100), anti-CD45-APC (Santa Cruz Biotechnologies; 1:100), and anti-CD146-FITC (AbD Serotec; 1:100). All incubations were performed at 4°C for 15 min in the dark. Before sorting, DAPI (Invitrogen; 1:1000) was added for dead cell exclusion, the solution was then passed through a 70-μm cell filter and then run on a FACSAria cell sorter (BD Biosciences). Sorted cells were plated at a density of twenty thousand cells per cm^2 in 0.2% gelatin-coated plates and cultured at 37°C in EGM2 (Lonza) for the first 2 weeks. Next, cells were cultured in DMEM high glucose, 20% fetal calf serum (FCS; Invitrogen), and 1% penicillin/streptomycin (BD Biosciences).

RT-PCR analysis

Total RNAs were extracted from a minimum of 2×10^4 freshly sorted or cultured cells by using Absolutely RNA nanoprep kit (Stratagene), and cDNAs were synthesized with SuperScript™ II reverse transcriptase (Invitrogen) according to manufacturer's instructions. PCR was performed with Taq polymerase (Invitrogen) per manufacturer's instructions for 35 cycles at 58°C annealing temperature, and PCR products were electrophoresed on 1% agarose gels.

Flow cytometry analysis

Cultured cells were labeled with the following commercial antibodies: anti-CD34-PE (BD Biosciences; 1:100), anti-CD44-PerCP-Cy5.5 (eBioscience; 1:100), anti-CD73-PE (BD Biosciences; 1:50), anti-CD90-APC (BD Biosciences; 1:50), anti-CD105-PE (Invitrogen; 1:50), and anti-CD146-PE (BD Biosciences; 1:100). Cells were then washed twice, and at least 50,000 events were acquired on an FACSCanto II cytometer (BD Biosciences). Each individual adventitial cell culture ($n=8$) was analyzed at different passages (p3 to p13).

Immunohistochemistry and immunocytochemistry

hWAT samples were impregnated in gelatin/sucrose, frozen in isopentane (Sigma) cooled in liquid nitrogen and embedded in tissue freezing medium (Triangle Biomedical Sciences). Five- to 9-μm sections were cut on a cryostat (Microm) and fixed for 5 min with 50% acetone (VWR International) and 50% methanol (Fischer Scientific). Sections were then dried at room temperature and washed thrice for 5 min in PBS. Nonspecific binding sites were blocked with 5% goat serum (Invitrogen) in PBS for 1 h at room temperature. Sections were incubated with uncoupled primary antibodies overnight at 4°C, or 2 h at room temperature in the case of directly coupled antibodies. After rinsing, sections were incubated for 1 h at room temperature with a biotinylated secondary antibody, then with fluorochrome-coupled streptavidin, both diluted in 5% goat serum in PBS. The same protocol was used for the staining of cultured cells at different passages. The following uncoupled anti-human primary antibodies were used: anti-CD146 (BD Biosciences; 1:100), anti-CD31 (DAKO; 1:100), anti-CD34 (BD Biosciences; 1:50), anti-CD44, anti-CD73, anti-CD105 (all from Invitrogen; 1:20), anti-CD90 (BD Biosciences; 1:20), anti-NG2 (1:100; BD Biosciences), and anti-PDGFR-β (1:50; Cell Signaling) and anti-vimentin (1:100; Sigma). Coupled antibodies used included the following: anti-α-SMA-FITC (Sigma; 1:100), anti-CD34-FITC (Miltenyi; 1:20), and anti-vWF-FITC (US Biological; 1:100). Human specific anti-dystrophin (Novocastra; 1:20) was used to detect dystrophin-positive myofibers in SCID/mdx mouse muscle sections. Secondary goat anti-mouse antibodies were biotinylated (DAKO; 1:500) or coupled to Alexa 488 (Molecular Probes; 1:500). Streptavidin-Cy3 (Sigma; 1:500) was used after the incubation with biotinylated secondary antibodies. Nuclei were stained with DAPI (4′, 6-diamino-2-phenylindole dihydrochloride, Molecular Probes; 1:2000) for 5 min at room temperature. An isotype-matched negative control was performed with each immunostaining. Slides were mounted in glycerol-PBS (1:1; Sigma) and observed and photographed on an epifluorescence microscope (Nikon Eclipse TE 2000-U).

Confocal microscopy analysis

Tissue sections were examined by confocal microscopy after multi-color immunofluorescence staining and DAPI nuclear staining. Human WAT frozen sections were stained with anti-CD34, -CD146, -α-SMA, or -vWF antibodies according to the protocol just described. Images were collected on an Olympus Fluoview 1000 confocal microscope with 40×, 60×, and 100× oil immersion objectives. Excitation wavelengths used for detection included 405, 488, 543, and 633 nm for DAPI, FITC, Cy3, and Cy5, respectively. Sequential detection was set to eliminate bleed-through artifacts between fluorophores.

Population doubling time calculation

Sorted cells cultured in DMEM + 20% FCS for 12 weeks were seeded into 6-well plates at a density of 2.0×10^3 cells/cm^2. Cells were grown for 120 h, and the population doubling time was calculated by using the formula: time/no. of doublings, where time = 120, and no. of doublings = log2 (N final/N initial). The assay was performed in biological and technical triplicates.

Differentiation into mesodermal cell lineages in vitro

For adipogenic differentiation, adventitial cells at 70% confluence were cultured in DMEM, 10% FCS, 1 μM dexamethasone, 0.5 μM isobutylmethylxanthine, 60 μM indomethacine, and 170 μM insulin (all from Sigma). After 14 days, cells were fixed in 2% PFA at room temperature, washed in 60% isopropanol, and incubated with Oil red O for 10 min at room temperature for detection of lipid accumulation. Cells at 70% confluence not cultivated in adipogenic differentiation medium were fixed and stained as described for use as a negative control.

For chondrogenesis, high-density pellets were prepared by spinning down 3×10^5 cultured cells in 15 mL conical tubes and grown in serum-free DMEM containing an insulin-transferrin-selenious acid mix (BD Biosciences), 50 μg/mL L-ascorbic acid 2-phosphate (Wako), 100 μg/mL sodium pyruvate, 40 μg/mL L-proline (both from Invitrogen), 0.1 μM dexamethasone (Sigma), and 10 ng/mL transforming growth factor β1 (TGF-β1) (Peprotech). Pellets cultured in the absence of TGF-β1 were considered as untreated and used as negative controls. After 21 days, pellets were fixed in 10% formalin, dehydrated using a graded series of ethanol washes, and embedded in paraffin. Five micrometers thick sections were rehydrated and stained with Alcian blue and nuclear fast red for the detection of sulfated glycosaminoglycans and nuclei, respectively.

For osteogenic differentiation, cells at 70% confluence were cultivated in osteogenic medium, consisting of DMEM, 10% FCS, 0.1 μM dexamethasone, 50 μg/mL L-ascorbic acid, and 10 mM β-glycerophosphate (all from Sigma). After 21 days, cells were fixed in 4% formaldehyde for 2 min and incubated for 10 min with alizarin red, pH 4.2 for the detection of calcium deposits. All the cell cultures ($n = 8$) were assayed for their developmental potential in technical triplicate.

Cell cloning

Clones were obtained by limiting dilution after plating cultured adventitial cells at a density of 1 cell/well in a 96 multi-well plate. Clones were grown from 2 different specimens at passages 5 and 10. A total of 5 plates (480-wells) were used for each cloning. Clones that reached confluence were expanded, and 5 individual clones obtained from each hWAT specimen were randomly selected and assayed for their phenotype and in vitro developmental potential as just described.

Differentiation into pericyte-like cells

Five thousand cultured adventitial cells were plated in 48-well plates in DMEM high glucose, 20% FCS, and 1% penicillin-streptomycin. When at 70% confluence, cells were cultured in DMEM high glucose, 0.5% FCS, 0.1 μM angiopoietin II (ANGPT2), and 1% penicillin-streptomycin for 72 h. Cells were then washed with PBS, fixed with cold methanol-acetone (1:1), and stained with anti-α-SMA, anti-CD146, anti-PDGFR-β, and anti-NG2 as described earlier. The assay was performed at different passages (p5 to p10) in biological duplicate and technical triplicate.

Statistical analysis

Results are expressed as mean ± SEM. Unpaired *t*-test was used to compare the means between 2 groups, and a P value < 0.05 was considered significant.

Results

MSC can be derived from adipose tissue CD34+ cells distinct from pericytes

We recently described primary MSCs within human organs as CD146+CD34-CD45-CD56- pericytes. Here, we set up to verify whether the whole potential to give rise to MSC in culture is confined within pericytes. To this end, we investigated the potential of a different sets of cells, distinct from pericytes and isolated from the stromal vascular fraction of hWAT, a well-documented source of adult multipotent cells, to give rise to MSC in culture. After exclusion of hematopoietic (CD45+) and dead (DAPI+) cells, 3 distinct populations could be detected by flow cytometry based on expression of CD34 and CD146. We isolated pericytes as CD146+CD34- cells (Fig. 1a, gray box), as well as a mixture of other cells differentially expressing CD34 and CD146 (Fig. 1a, black box). Outgrowth of MSCs was observed from both cultured populations. As shown in Fig. 1b, we then separated the 2 nonpericyte subpopulations defined as CD34+CD146- and CD34+CD146+. Cultivated CD34+CD146+ cells never gave rise to MSC, whereas MSC-like cells arose from CD34+CD146- cells in vitro. The endothelium-specific antigen CD31 was detected in only CD34+CD146+ cells; thus, no endothelial cells are present within CD34+CD146- progenitors of MSC (Fig. 1c). Long-term cultures of CD34+CD31-CD146- cells, representing 9.8 ± 1.7% of the total stromal vascular fraction, were successfully established as MSC from all specimens processed for this purpose ($n = 12$). To exclude the possibility of a contamination by pericytes, the purity of CD34+CD31-CD146- cells was verified immediately after the sort by RT-PCR analysis, and no CD146 expression was ever detected (Fig. 1d). Further, sorted cells expressed CD34 but not CD31 or CD144 (VE-cadherin), thus confirming that the purified CD34+CD31-CD146- cells are distinct from pericytes and endothelial cells.

CD34+ CD146- cells are presented in multiple organs

We next investigated whether this novel population of MSC progenitors is hWAT specific or rather present in multiple organs. Using the sorting strategy just described for hWAT, we detected the same population of CD34+CD146- cells also in fetal muscle, lung, and bone marrow (Fig. 2a–c). MSC-like cells were obtained in cultures regardless of the tissue of origin, thus demonstrating that CD34+CD146- cells are MSC progenitors distinct from pericytes distributed in different tissues.

CD34+ CD146- cells display MSC features at the clonal level

MSC are culture-established clonogenic progenitors characterized by a distinct surface phenotype and the ability to differentiate into mesodermal cell lineages. To investigate whether CD34+CD146- cells meet the criteria for being MSC ancestors, we cloned by limiting dilution CD34+CD146- cells obtained from 2 different hWAT samples. Clones developed with an efficiency of 15.6% ± 1.5%, with differences in proliferation potentials, which suggested that CD34+CD146- cells

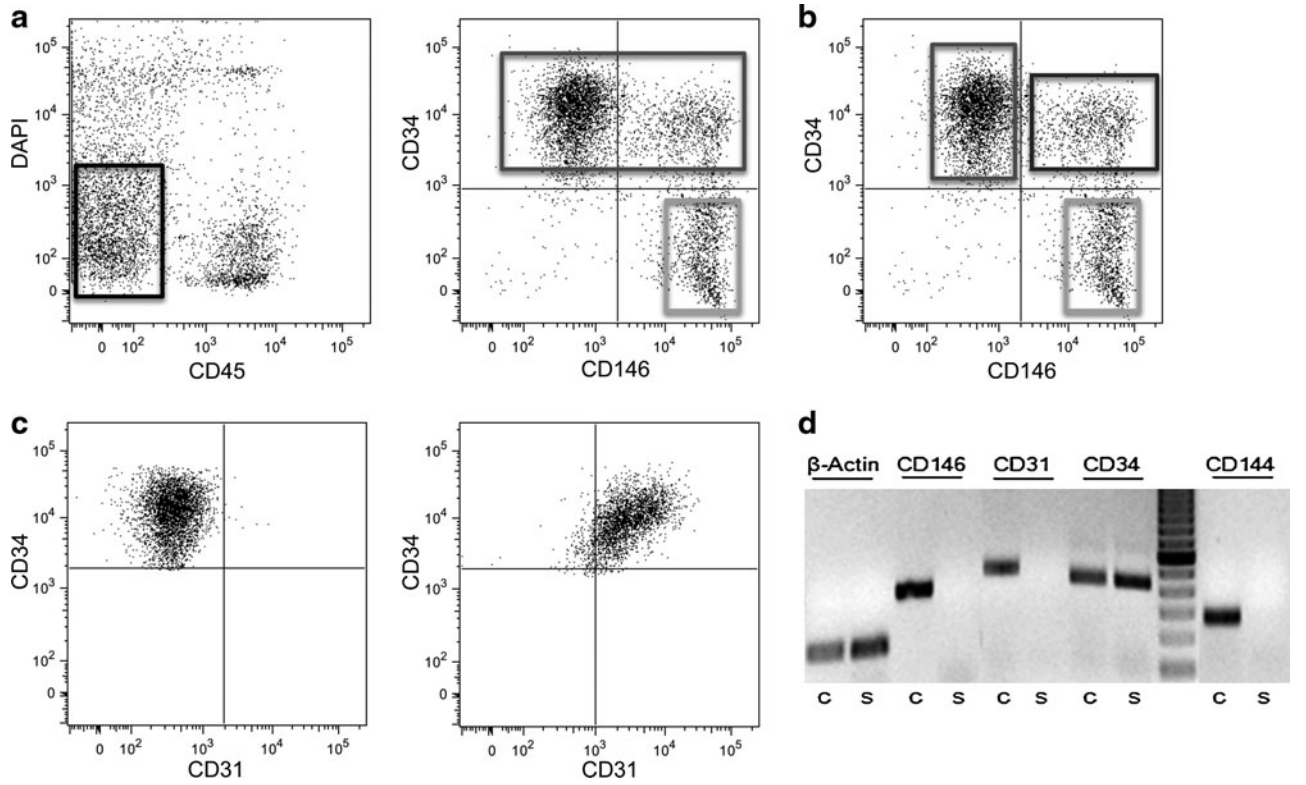

FIG. 1. Purification of nonpericyte MSC-like cells from human adipose tissue. **(a)** After exclusion of DAPI+ dead cells and CD45+ hematopoietic cells, pericytes were isolated as CD146+CD34- cells (*gray gate*) as previously described. MSC-like cells could also be grown from the mixed population of nonpericyte cells differentially expressing CD34 and CD146 (*black gate*). **(b)** The following nonpericyte cell populations were then purified: CD34+CD146-, CD34+CD146+ and cultured in MSC conditions. The outgrowth of MSC-like cells was observed only after culture of CD34+CD146- cells. **(c)** Analysis of CD31 expression on gated CD34+CD146- cells (*left dot plot*) and CD34+CD146+ cells (*right dot plot*). CD31 was detected in only endothelial CD34+CD146+ cells, whereas no endothelial cells were detected within CD34+CD146- cells. **(d)** RT-PCR analysis was performed to check the purity of sorted CD34+CD31-CD146- cells. CD34 but no CD31, CD144, and CD146 mRNA was detected, thus excluding a possible contamination by endothelial cells or pericytes. C, positive control (unfractionated stromal vascular fraction); S, freshly sorted CD34+CD146- cells. The same results were observed in 3 distinct experiments. MSC, mesenchymal stem cell.

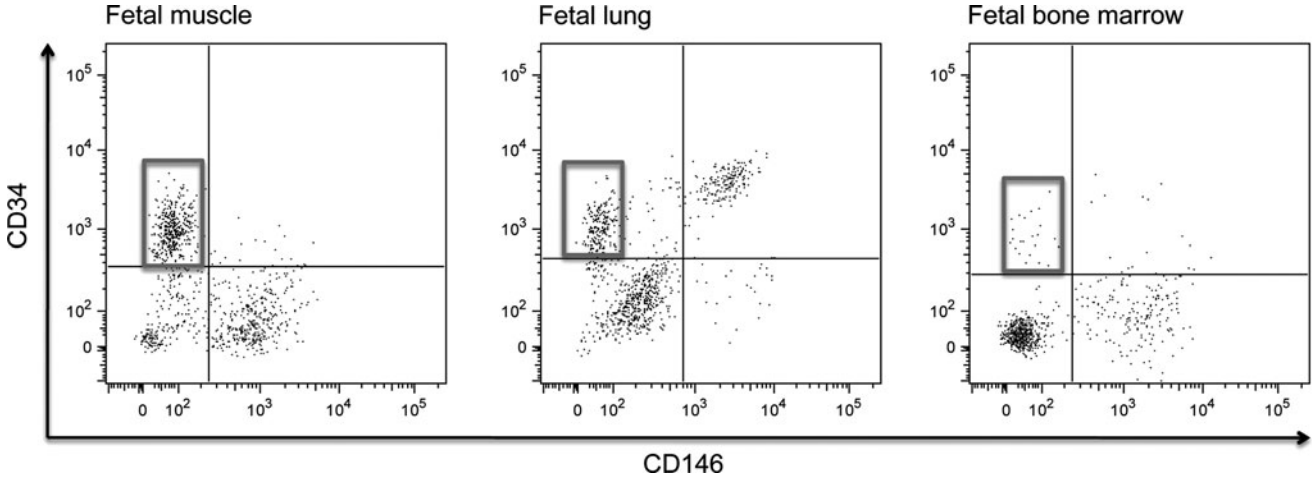

FIG. 2. CD34+CD146- cells are present in multiple organs. Using the same sorting strategy described for the isolation of MSC-like cells from WAT, CD34+D146- cells were detected in other tissues such as fetal muscle, lung, and bone marrow (from left to right). MSC-like cells were obtained from CD34+CD146- cells regardless of the tissue of origin. WAT, white adipose tissue.

are heterogeneous. Only 2.7%±1.7% of the clones formed could be serially passaged and further characterized (Fig. 3a). All clones displayed the MSC hallmark markers CD44, CD73, CD90, and CD105 at every passage tested, up to passage 10 (Fig. 3b). All clones were also able to differentiate in vitro into adipocytes, osteocytes, and chondrocytes, as shown in Fig. 3c–e. No significant differences in phenotype and developmental potential were observed at different passages in polyclonal cultures (p3-p10) or among different clones. We, therefore, established that CD34+ CD146- cells are genuine progenitors of MSC. The same phenotype and developmental potential was observed in adventitial cells isolated from fetal bone marrow and lung (data not shown).

Native distribution of CD34+CD146- cells in human tissues

Immunohistochemistry was performed on sections of WAT, adult and fetal muscle, adult pancreas, and fetal lung to uncover the natural localization of CD34+CD146- cells. We previously confirmed that, in capillaries (diameter: 8–10 μm), CD34 is exclusively expressed by endothelial cells [15]. In the current study, the only cells expressing CD34, but not CD31 and CD146, consistent with the FACS plot shown in Fig. 1c (left panel), were located around blood vessels larger than capillaries, spanning arterioles, venules, arteries, and veins, with a diameter greater than 50 μm. Together with CD34, we used markers to detect endothelial cells (CD31) and pericytes/smooth muscle cells (CD146 and α-SMA). Double staining confirmed the co-expression of CD34 and CD31 by all endothelial cells. Conversely, cells located in the outmost layer of vessel walls express CD34 but not CD31 (Fig. 4a–c). Double stainings of CD34 and CD146 or α-SMA were then performed to localize nonendothelial CD34+ cells. As shown in Fig. 4d, e, α-SMA+CD146+smooth muscle cells are surrounded by CD34+ cells. No expression of CD34 by pericytes/smooth muscle cells was ever observed, thus confirming our previous observations [15]. By triple staining and confocal microscopy analysis, we have typified the cells that constitute the 3 layers of small to large vessel walls as CD34+CD146-α-SMA- adventitial cells, CD146+α-SMA+

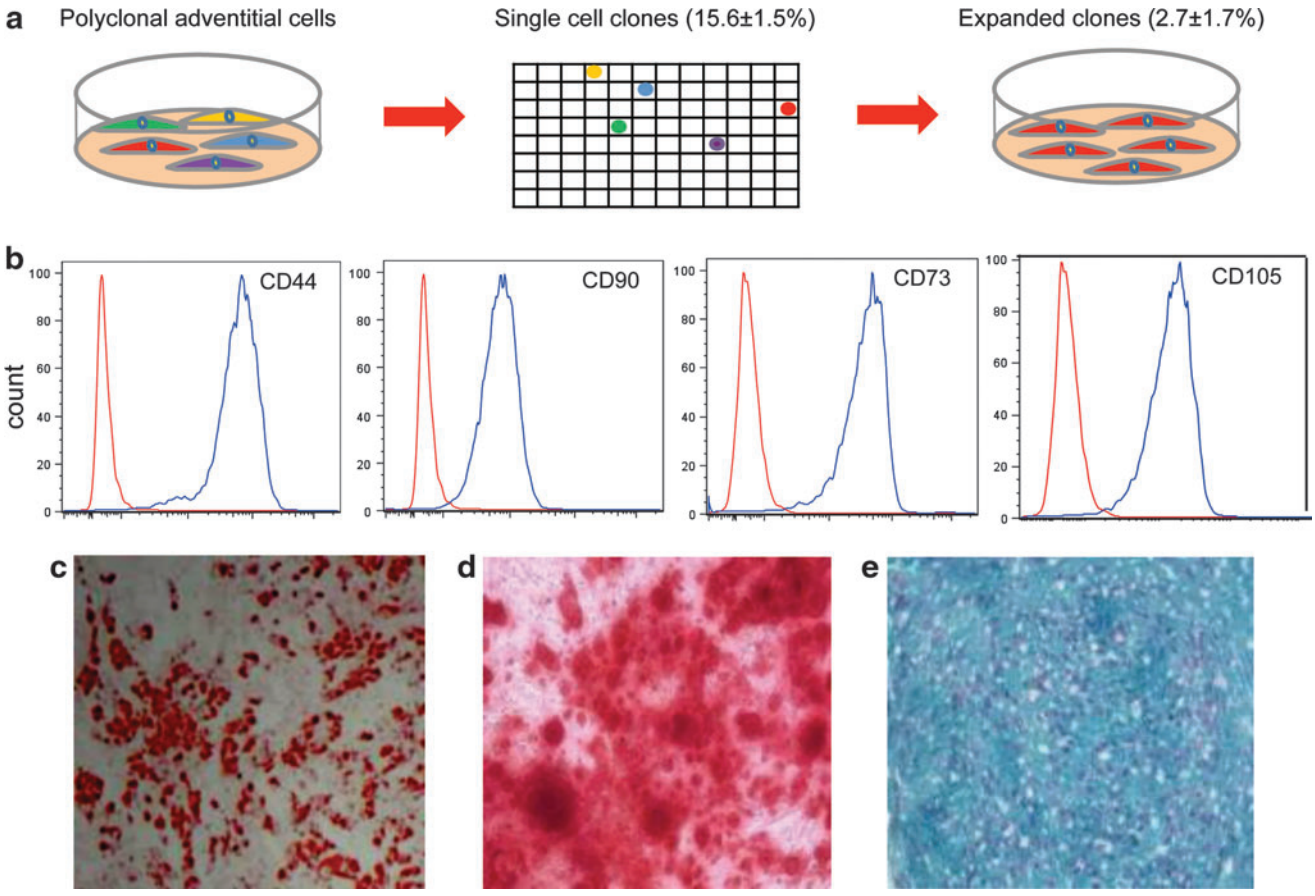

FIG. 3. Cultured CD34+CD146- cells are clonogenic multipotent progenitors similar to MSC Representative characterization of a clone at late passage of culture (p10). The phenotype and developmental potential are maintained throughout culture with no significant change after long-term culture. **(a)** Polyclonal cultured CD34+CD146- cells were cloned by limiting dilution. Only a minority of the single-cell clones could be expanded for further characterization. **(b)** Single cell-derived clones homogeneously express typical MSC markers (CD105, CD44, CD90, and CD73). *Red line*: unstained negative control sample; *blue line*: stained sample. **(c–e)** Single-cell clones can each differentiate into several mesodermal cell lineages when cultured under specific conditions. Oil red O, alizarin red and alcian blue staining reveal differentiation into adipocytes **(c)**, osteocytes **(d)**, and chondrocytes **(e)**, respectively. Color images available online at www.liebertonline.com/scd

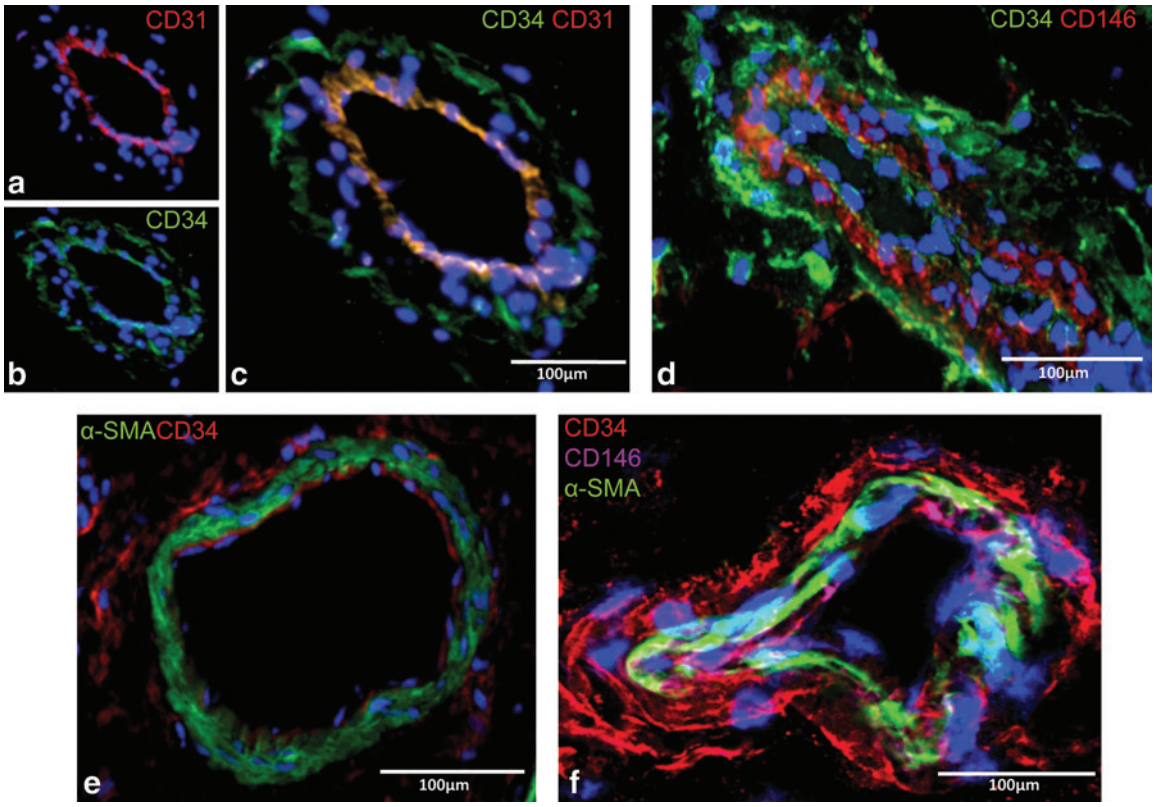

FIG. 4. In situ localization of CD34+CD31-CD146- cells. Immunohistochemistry was performed on hWAT frozen sections. In vessels with a lumen diameter greater than 50 μm, CD31 is specifically expressed by endothelial cells in the tunica intima **(a**, 20×**)**, whereas CD34 is expressed by 2 distinct populations of differently located cells **(b**, 20×**)**. As shown by merging the 2 stainings, CD34+CD31+ cells are endothelial, whereas a subset of CD34+CD31- cells is located in the outer layer of the blood vessel **(c**, 20×**)**. Double staining of CD34 and pericyte/smooth muscle cell markers CD146 **(d**, 20×**)** or α-SMA **(e**, 20×**)** reveals that nonendothelial CD34+ cells are located in the tunica adventitia surrounding smooth muscle cells. Multi-color staining was performed with antibodies against CD34 (*red*), CD146 (*purple*), and α-SMA (*green*) to identify the 3 subsets of cells constituting the vessel wall **(f**, 60×**)**. Confocal microscopy confirmed that CD34-positive cells residing in the vascular adventitia do not co-express CD146 or α-SMA. In contrast to adventitial cells, smooth muscle cells surrounding endothelial cells express both CD146 and α-SMA. Endothelial cells are identified by their luminal localization and co-expression of CD34 and CD146 **(f**, 60×**)**. Color images available online at www.liebertonline.com/scd

CD34- pericytes/smooth muscle cells, and CD34+CD146+/-α-SMA- endothelial cells (Fig. 4f). In conclusion, the anatomic candidate marked as CD34+CD31-CD146- is a vascular adventitial cell.

Adventitial cells express MSC markers prior to ex vivo culture

Flow cytometry analysis on freshly dissociated adipose tissue revealed that stromal vascular fraction derived adventitial cells natively and homogeneously express the typical MSC markers CD44, CD73, CD105, and CD90 (Fig. 5a). Immunohistochemical staining of serial sections from the same adipose tissue confirmed that, besides microvascular pericytes, the only other cells coexpressing CD44, CD90, CD73, and CD105 are, indeed, distributed in the tunica adventitia of larger blood vessels, surrounding α-SMA+ smooth muscle cells (Fig. 5b–e). Altogether, these results indicate that adventitial cells are native progenitors of MSC, anatomically distinct from pericytes and peripheral to blood vessels larger than arterioles and venules.

Cultured adventitial cells treated with a vessel remodeling growth factor acquire pericyte traits

The observation that adventitial cells and pericytes are anatomically and phenotypically different, yet all give rise to *bona fide* MSC in culture, prompted us to closely and comparatively follow both cells along extended in vitro expansion. Despite similar morphologies, calculation of population doubling times (PDT) after 12 weeks revealed that adventitial cells proliferate significantly faster than pericytes (Fig. 6a). As of surface phenotype, adventitial cells isolated from 8 distinct hWAT samples expressed no CD146 at any passage tested (up to p13), whereas all cultured pericytes did (Fig. 6b), as already reported [15]. No expression of CD34 was detected in either population by flow cytometry (data not shown). As previously demonstrated [15], human pericytes express PDGFR-β, CD146, α-SMA, and NG2 (Fig. 6d–g), none of which was ever detected in adventitial cells cultured in regular conditions (Fig. 6i–l), in which the 2 cell types only shared the expression of vimentin (Fig. 6h, m). However, treatment with 0.1 μM ANGPT2 for 72 h induced expression

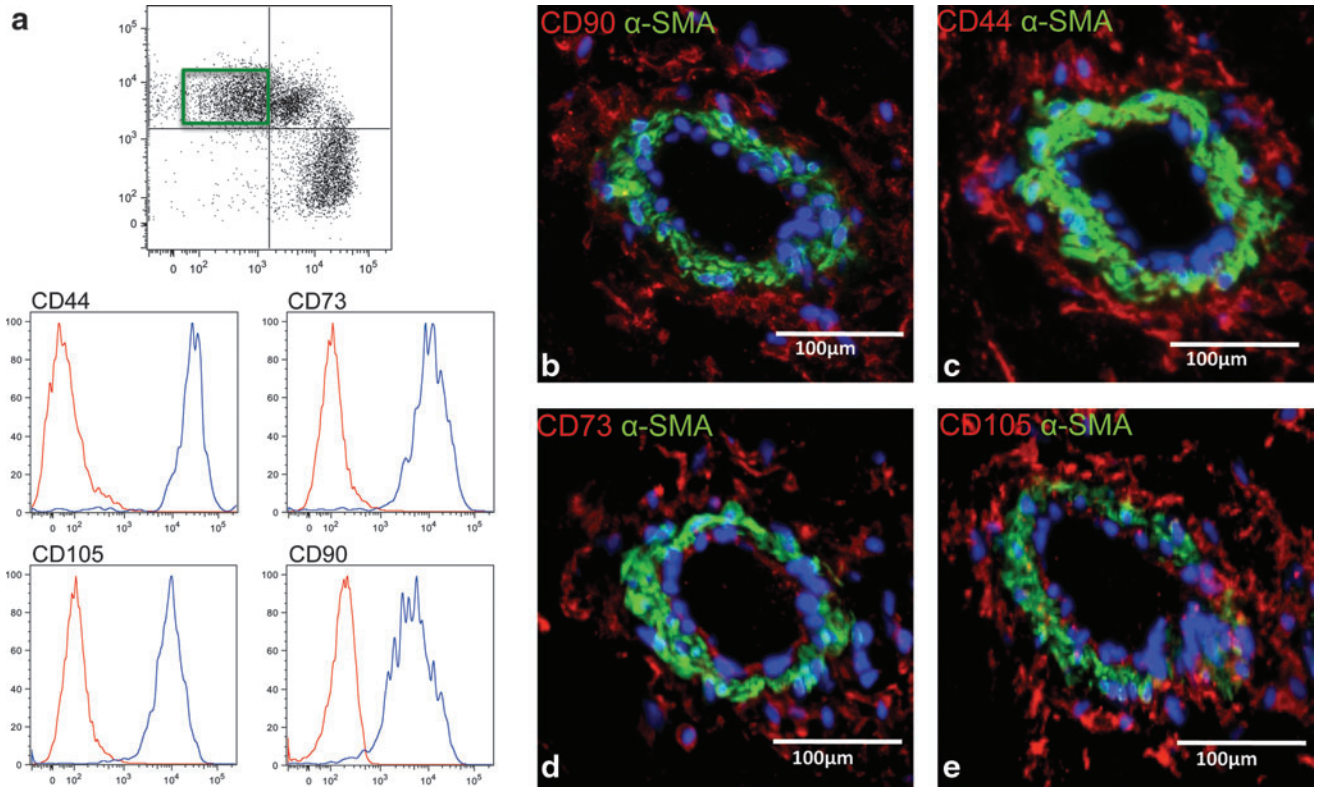

FIG. 5. Adventitial cells express typical MSC markers before *ex vivo* expansion. **(a)** Flow cytometry analysis was performed on fresh stromal vascular fraction before culture ($n=3$). The expression of hallmark MSC markers was detected within the subset of adventitial cells, previously defined as CD34+DAPI-CD45-CD31-CD146- (*green box*). Adventitial cells homogeneously express all the MSC markers tested. *Red histogram line:* unstained cells. *Blue histogram line:* stained cells. **(b–e)** Serial staining of an adipose tissue artery with antibodies to α-SMA and MSC markers. All markers tested, CD90 **(b)**, CD44 **(c)**, CD73 **(d)**, and CD105 **(e)**, are co-localized in the cell layer surrounding α-SMA+smooth muscle cells, thus confirming that CD34+CD31-CD146- multipotent progenitors reside in the tunica adventitia. As expected, CD105 and CD73 were also expressed in endothelial cells **(d, e)**. Color images available online at www.liebertonline.com/scd

of all the aforementioned markers in cultured adventitial cells, thus suggesting differentiation into pericyte-like cells (Fig. 5n–q).

Discussion

Historically, MSCs were isolated on their capacity to selectively proliferate in cultures of total bone marrow and to differentiate in mesodermal cell lineages. Some authors have also described MSC differentiation into nonmesodermal cell lineages, although this is still a matter of debate [30,31]. Remarkably though, MSC did not benefit the theoretical and technologic progress that permitted the prospective identification of hematopoietic and other stem cells. The instrumentalization of MSC for tissue-engineering/cell therapy eclipsed latent questions regarding the identity, origin, and native distribution of these adult stem cells. Only recently were similarities noticed to exist between MSC and perivascular cells [10–14]. Indeed, vascular pericytes purified to homogeneity from multiple organs express MSC markers, can differentiate into mesoderm lineage cells, and give rise in culture to genuine MSC [15]. Are all MSC derived from pericytes, which are exclusively associated with capillaries and microvessels [16]? The current work addressed this issue along a substractive approach, by investigating the ability of FACS selected nonpericyte cells, within the stromal vascular fraction of hWAT, to give rise to MSC in culture. Our general conclusion is that CD34+CD31-CD146-CD45- cells residing in the outer layer (*tunica adventitia*) of arteries and veins yield in culture multipotent progenitors functionally and antigenically similar to MSC. Coincidentally, the *tunica adventitia* is believed to play a role in vascular remodeling and be involved in the development of atherosclerosis [17–20]. Adventitial cells indeed proliferate, differentiate into myofibroblasts, and migrate into the inner layer of blood vessels in response to injury or stress [21–25]. CD34+CD31- cells located in the "vasculogenic zone" of the thoracic artery, between the *tunica media* and *tunica adventitia*, can also give rise to endothelium; hence, they could represent a resident pool of endothelial progenitor cells for postnatal vasculogenesis [26]. Multipotent progenitors can originate from larger vessels not surrounded by typical pericytes, such as the human pulmonary artery, fetal aorta wall, and vena saphena [27–29], thus further supporting our present conclusions.

The presence in hWAT of CD34+ MSC progenitors has been reported, although the native identity of these cells has been controversial so far. Traktuev et al. described in adipose tissue CD34+CD31- perivascular cells that, once enriched by plastic adherence and briefly cultured, expressed markers of pericytes (PDGFR-β), MSC (CD90), and smooth muscle cells

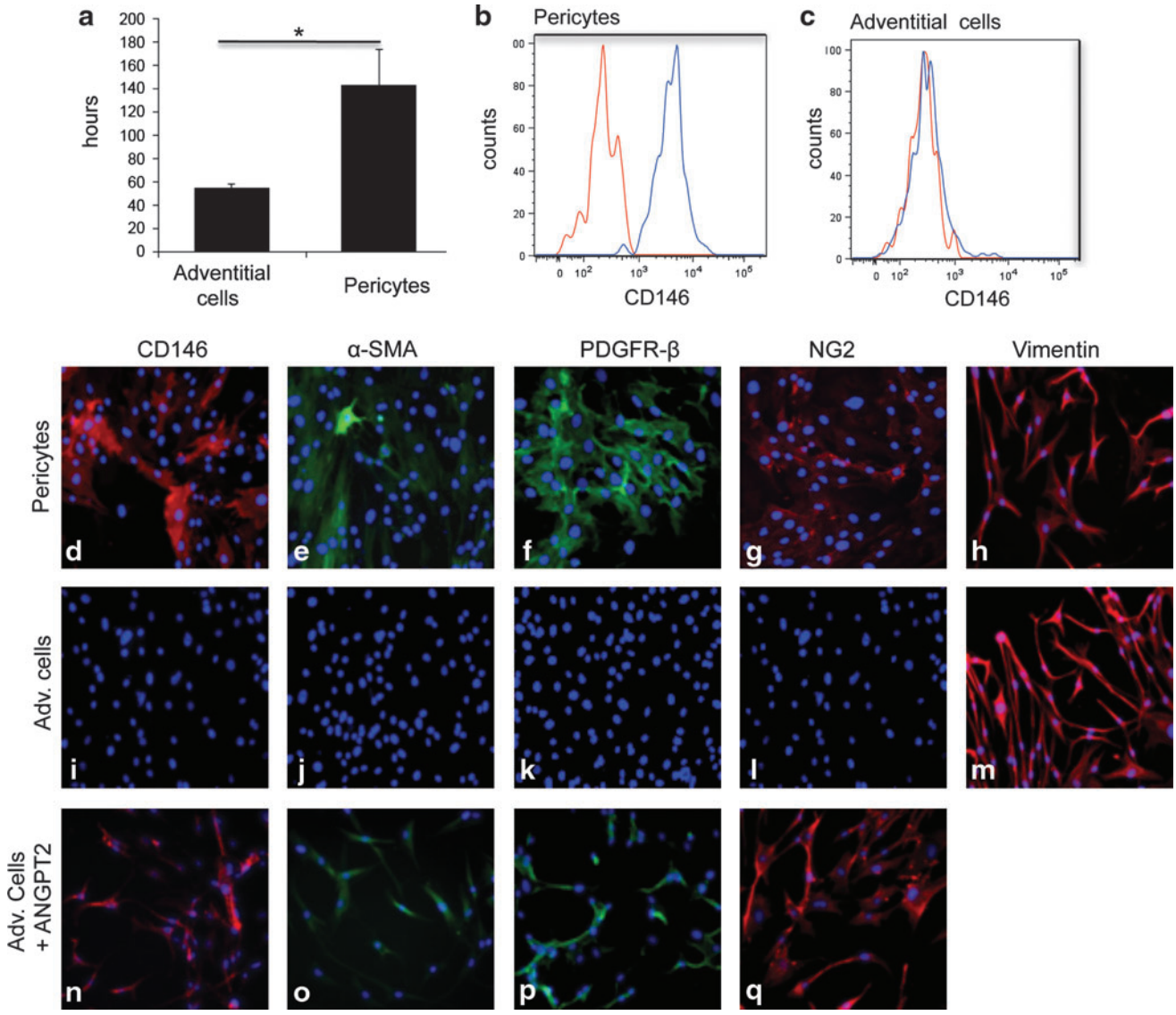

FIG. 6. In vitro characterization of long-term cultured adventitial cells **(a)** After 12 weeks of culture, adventitial cells show a significantly lower population doubling time (PDT) compared with pericytes (*$P < 0.05$) **(b, c)** At difference with pericytes, cultured adventitial cells never express CD146. *Red line*: unstained negative control sample; *blue line*: stained sample. **(d–q)** As previously reported, cultured pericytes express CD146 **(d)**, α-SMA **(e)**, PDGFR-β **(f)**, and NG2 **(g)**. None of these markers is expressed by cultured adventitial cells **(i–l)**. The 2 cell types only share expression of vimentin **(h, m)**. When exposed to 0.1 μM angiopoietin 2 (ANGPT 2) for 72 h, adventitial cells acquired all pericyte markers: CD146, α-SMA, PDGFR-β, and NG2 **(n–q)**. Color images available online at www.liebertonline.com/scd

(α-SMA) [32]. In 2 other studies, CD34+CD31- cells were clearly identified in human adipose tissue as adventitial cells distinct from α-SMA+ smooth muscle cells and PDGFR-β+ or CD146+ pericytes, all of which are invariably CD34 negative [33,34]. Adventitial cells and pericytes from fat, therefore, include 2 anatomically and phenotypically distinct perivascular primary MSC, an observation we have extended to human fetal lung, fetal and adult pancreas, and adult muscle. Importantly, no potential to generate MSC was observed, in any tissue analyzed, outside these 2 perivascular cell compartments. Adventitial fibroblasts can differentiate into α-SMA+ myofibroblasts under hypoxia, or when exposed to growth factors involved in vascular remodeling and angiogenesis, TGF-β or angiotensin II [35–37]. Consistently, adventitia-derived MSC treated with angiotensin II upregulate the expression of α-SMA, which typifies fibroblasts, pericytes, smooth muscle cells, and myofibroblasts. We also show for the first time that adventitial cells cultured in the presence of angiotensin II upregulates other pericyte markers: CD146, PDGFR-β, and NG2. The same outcome was observed when adventitial cells were treated with angiopoietin-2, a growth factor produced at the site of vascular remodeling that disrupts interactions between smooth muscle cells and endothelial cells, thus leading to angiogenesis in the presence of VEGF [38]. Altogether, our results indicate the ability of adventitial cells to differentiate into pericytes-like cells under appropriate signaling, thus supporting the previously hypothesized existence of CD34+CD31- pericyte

progenitors [39,40]. Differentiation into myofibroblasts suggests that adventitial cells are precursors of smooth muscle cells in homeostasis and disease, in agreement with the emerging theory of vascular remodeling "from the outside-in," as the adventitia being the sensor of vascular injury or other environmental stress [41]. Given the observed capacity of adventitial cells to differentiate into MSC and pericyte-like cells, here we propose a hypothesis: the centrifugal migration of multipotent adventitial progenitors to surrounding regenerating tissues, in particular at the developing blood vessels.

In final conclusion, we have demonstrated that human blood vessels, depending on their size, are associated with 2 distinct peripheral MSC progenitors, namely pericytes encircling capillaries and microvessels [15], and adventitial cells surrounding larger arteries and veins. This hypothesis of a dual origin for MSC is also supported by the presence of both CD146+ cells (40%–60%) and CD146- cells within MSC conventionally derived in culture [13]. We propose that, in hWAT and presumably all organs, vascular progenitors of MSC are hierarchically organized with adventitial cells being precursors of pericytes, based on their higher proliferation rate and potential to differentiate into pericytes on proper stimulation.

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Author Disclosure Statement

No competing financial interests exist.

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